METHODS AND COMPOSITIONS FOR TREATING MICROTUBULE-MEDIATED VIRAL INFECTIONS AND LESIONS

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PRIORITY CLAIM

This application is a continuation of Patent Application Serial Number 09/506,415-filed February 17, 2000, and claims benefit under 35 U.S.C. § 119(e) of prior Provisional Patent Application Serial Number 60/120,582-filed February 18, 1999.

FIELD OF THE INVENTION

This invention relates generally to treatment methods and compositions comprising naturally occurring or synthetic compounds that interfere with the normal structure and function of tubulin and the normal formation of microtubule structures within a host cell. The treatment methods are for preventing or treating virus infections in a mammal, and the dermal or mucosal lesions or tumors associated with certain viral infections in a mammal, that are dependent upon the microtubule-mediated cytoplasmic transport of a viral genome within the host cell, or otherwise exploits microtubule dynamics within the host cell.

BACKGROUND OF THE INVENTION

Microtubules are one of the primary elements of the cytoskeletal structure of virtually all eukaryotic cells. Microtubules are hollow polarized cylinders of approximately 25 nm constructed of protein heterodimers of α-tubulin and β-tubulin and are typically anchored to a microtubule-organizing center (MTOC) located near the cell nucleus. They have a dynamic fast-growing "plus-end" and a slower growing "minus-end". The tubulin sub-units are moved

toward the minus-end of the microtubule when additional tubulin sub-units polymerize at the plus-end, thus creating a "treadmilling" effect.

Microtubule associated proteins (MAPs) are involved in the movement of materials across the cytoplasm. Dynein is a minus-end directed MAP, and kinesin is a plus-end directed MAP. Dynein is associated with the movement of materials across the cytosol from the cell membrane toward the nuclear pore complex. One domain of the dynein MAP attaches to the cargo and another domain attaches to a tubulin sub-unit. As the tubulin sub-unit moves toward the minus-end of the microtubule, it carries the cargo along with it. Kinesin is associated with the movement of materials across the cytosol from the nucleus toward the cell membrane.

Viral genomes are one example of materials that are transported across the cytoplasm of a host cell by normally functioning microtubule dynamics. For example, recent studies investigating the mechanisms of viral transport in the cytoplasm demonstrate that herpesvirus replication is dependent on the normal functioning of microtubule dynamics within the host cell. An incoming HSV capsid binds to the microtubule at the plus end as described herein and is transported toward the minus-end where it is released and enters the cell nucleus to begin the replication process.

These observations suggest a novel approach to the development of effective antiviral therapies comprising pharmacological agents that interfere with the normal structure or function of microtubules within mammalian cells. To the best of the applicant's knowledge, such antiviral therapies have not heretofore been identified or described.

Researchers have observed that a number of viruses are dependent upon microtubule-mediated cytosolic transport for replication. Examples of these may include, but not limited to, Herpes Simplex 1 (HSV-1), Herpes Simplex 2 (HSV-2), Cytomegalovirus (CMV), Varicella-Zoster virus (VCV), Epstein Barr virus (EBV), Herpes Simplex 6 (HSV-6), Herpes Simplex 7 (HSV-7), Herpes Simplex 8 (HSV-8), Papilloma virus (PPV), Vaccinia virus (VV), Adenovirus, Parvovirus, Human Immunodeficiency virus (HIV), and rabies virus.

Examples of compounds that have been identified as anti-microtubule agents include, but are not limited to, taxanes and taxoids, discodermolide, epothilones A and B, eleutherobin, taccalonolide, colchicine, colcemid, demecolcine, the vinca alkaloids including vincrisitine, vinepidine, vindesine, vinblastine, vinorelbine, desformyl vincrisitine, desacetyl desformyl vincristine, and vinflunine, phomopsin A, ustiloxins, cryptophycins, halichondrins, estramustine, rhizoxin, nocodazole, and any analogues or derivatives of any of the above.

Taxanes (e.g., paclitaxel, docetaxel), discodermolide, epithilones A and B, eleutherobin, and taccalonolide, are examples of a novel class of anti-microtubule agents that

share the ability to stabilize microtubules by inducing tubulin polymerization and inhibiting microtubule disassembly.

Paclitaxel (also known under the trademark Taxol®) is perhaps the most familiar of the taxanes. It was first isolated in 1971 from the bark of *Taxus brevifolia*, commonly known as the Pacific Yew, and was approved in 1992 by the US Food and Drug Administration for treatment of metastatic ovarian cancer and later for breast cancer. Its mechanism of action is believed to involve promoting formation and hyperstabilization of microtubules, thereby preventing the disassembly of microtubules necessary for completion of cell division. It also has been reported that paclitaxel induces expression of cytokines, affects the activity of kinases and blocks processes essential for metastasis, in as yet uncharacterized mechanisms of action.

Paclitaxel has attracted unusually strong scientific attention, not only because of its unique antiproliferative mechanism of action, but also because it is active against nearly all cancers against which it has been tested, and because it has been discovered to be an analog of numerous, closely-related compounds occurring naturally. Taxanes are now recognized as an important new class of anticancer compounds.

Eleutherobin was originally discovered in rare soft corrals belonging to the family *eleutherobia* collected from a specific region of the Indian Ocean near Australia. It has since been synthesized by scientists at The Scripps Research Institute. Eleutherobin has a mechanism of action similar to paclitaxel, i.e., the stabilization of microtubules and the inhibition of microtubule disassembly.

Epithilones were originally isolated from a species of soil bacteria collected from the banks of the Zambezi River in the Republic of South Africa, and researchers at The Scripps Research Institute have synthesized derivatives of these compounds. Epothilones are of particular interest because they are more soluble in water than paclitaxel, have higher activity, and are more easily available (i.e., from a cellulose degrading bacterium). They have been shown to displace paclitaxel from its binding site in β-tubulin, and have demonstrated a mechanism of action similar to that of paclitaxel.

Discodermolide was originally isolated from the sponge Discodermia dissoluta and has now been totally synthesized. Discodermolide binds to tubulin dimers in microtubules and induces the polymerization of tubulin similar to paclitaxel, and is perhaps even more potent as anti-microtubule agent.

Colchicine, colcemid, demecolcine, the vinca alkaloids including vincrisitine, vinepidine, vindesine, vinblastine, vinorelbine, desformyl vincrisitine, desacetyl desformyl vincristine, and vinflunine, phomopsin A, ustiloxins, cryptophycins, halichondrins,

estramustine, rhizoxin, and nocodazole are examples of a novel class of anti-microtubule agents that share the ability to interfere with normal microtubule dynamics by inhibiting the polymerization of tubulin within a host cell, thus preventing the formation of microtubules.

Colchicine is a water-soluble alkaloid found in the autumn crocus. The vinca alkaloids, vinblastine and vincristine, are derived from the Madagascar periwinkle, and vindesine and vinorelbine are semisynthetic derivatives of vinblastine. These alkaloids also apparently interfere with a cell's ability to synthesize DNA and RNA. The ustiloxins and phomopsins are a family of tubulin-binding cyclic peptides, which have shown potent in vitro anti-tumor activity, particularly against human breast and lung cancer cell lines. Rhizoxin is produced by the fungus *Rhizopus chinensis*, and has also demonstrated the ability to inhibit the polymerization of tubulin into microtubules.

Thus, there is an unmet need to have an anti-viral treatment repertoire using a plurality of compositions against a plurality of viruses that target microtubule related processes.

SUMMARY OF THE INVENTION

The invention provides the treatment repertoire using effective amounts of at least one composition of the plurality of compositions that target microtubule related processes that are introduced to mammals via parenteral, oral, nasal, anal, aural, ocular, and topical routes of administration. The parenteral routes of administration includes intervascular injections, intermuscular injections, interdermal injections, subdermal injections, interspinal injections, and intercerebral injections. The intervascular injections further include intravenous and interarterial injections.

The invention provides formulations comprising an effective amount of one or more pharmacological agents known to interfere with the normal structure or function of microtubules. The invention describes an application of these formulations to mammalian cells, either topically or systemically, for the purpose of preventing or treating viral infections and the dermal or mucosal lesions or tumors associated with viral infections.

The present invention provides compositions and methods for treating diseased, biological tissue, such as the epidermis, in mammals. The plurality of compositions of the present invention can be used to treat epidermal lesions, such as those resulting from viral infections including, but not limited to: HSV-1, HSV-2, HSV-6, HSV-7, HSV-8, VZV, CMV, EBV, and PPV. The plurality of compositions of the present invention can also be used to treat epidermal lesions, ulcerations, abrasions, inflammation and other conditions resulting from microbial infections. In particular, compositions of the present invention are especially adapted to treat lesions caused by herpes viruses.

The effective amounts of at least one of the compositions of a plurality of compositions applied to a mammal is determined by assessing whether virus reductions in virus infections are manifested as improvements in clinical signs presented by the mammal, and re-applying the effective amount until reduction in virus infections are manifested as improvements in clinical signs presented by the mammal. How compositions are applied depend upon the intended route of administration used in introducing the effective amounts of compositions that target microtubule related processes. The effective amounts depends upon composition stabilities, concentrations, and solubilities, and whether single or more than one composition is administered via parenteral, oral, anal, aural, nasal, ocular, and topical routes of administration.

In one embodiment, the present invention provides compositions useful for treating diseased, biological tissue, such as the epidermis, in mammals. The compositions of the present invention are effective in treating viral infections and inflammation and lesions associated with viral infections. The compositions of the present invention include at least one anti-microtubule agent. The anti-microtubule agent can be a naturally occurring compound, a semi-synthetic compound, or can be an entirely synthetic compound that is chemically synthesized by any means known to those skilled in the art.

Examples of compounds that have been identified as anti-microtubule agents include, but are not limited to, taxanes, taxoids, discodermolide, epothilones A and B, eleutherobin, taccalonolide, colchicine, colcemid, demecolcine, the vinca alkaloids including vincrisitine, vinepidine, vindesine, vinblastine, vinorelbine, desformyl vincrisitine, desacetyl desformyl vincristine, and vinflunine, phomopsin A, ustiloxins, cryptophycins, halichondrins, estramustine, rhizoxin, and nocodazole. It is appreciated that the examples of compounds include any analogues or derivatives of the foregoing anti-microtubule agents.

BRIEF DESCRIPTION OF THE DRAWINGS

The preferred and alternative embodiments of the present invention are described in detail below with reference to the following drawings.

The foregoing aspects of many of the attendant advantages of this invention will become more readily appreciated as the same becomes better understood by reference to the following detailed description, when taken in conjunction with the accompanying drawings, wherein:

FIGURE 1A is a Drug Screening Report for TBT which lists the analytical results for bioactive effectiveness of TBT in terms of CPE, EC_{50}, CC_{50}, IC_{50}, SI = CC_{50}/EC_{50};

Cytopathic Effect Inhibition and Plaque Reduction Assays For HSV-1, HSV-2, CMV, MCMV, and VSV, in HFF cell cultures are detailed;

FIGURE 1B is a Drug Screening Report for TBT which lists the analytical results for bioactive effectiveness of TBT in terms of CPE, EC_{50}, CC_{50}, IC_{50}, SI = CC_{50}/EC_{50}; Cytopathic Effect Inhibition and Plaque Reduction Assays For EBV in DAUDI cell cultures are detailed; Toxicity Assays in HFF and DAUDI cell cultures are detailed;

FIGURE 2 is a descriptive listing of the HSV ELISA Method procedural steps used in measuring the anti-viral effectiveness of TBT to various HSV-1 and HSV-2 strains;

FIGURE 3 represents the analytical IC_{50} results for HSV-1 viral antigens, specifically, for HSV-1 control (ATCC #VR-733, Strain F), which demonstrated a TBT-IC_{50} = 3 μg/ml;

FIGURE 4 represents the analytical IC_{50} results for HSV-1 viral antigens, specifically, for HSV-1, clinical specimen #1 (TBT-IC_{50} = 3 μg/ml);

FIGURE 5 represents the analytical IC_{50} results for HSV-1 viral antigens, specifically, for HSV-1, clinical specimen #2 (TBT-IC_{50} = 0.25 μg/ml);

FIGURE 6 represents the analytical IC_{50} results for HSV-2 viral antigens, specifically for HSV-2 control (ATTC #VR-734, Strain G), which demonstrated a TBT-IC_{50} = 0.75 μg/ml;

FIGURE 7 represents the analytical IC_{50} results for HSV-2 viral antigens, specifically for HSV-2, clinical specimen #3 (TBT-IC_{50} = 2.0 μg/ml);

FIGURE 8 represents the analytical IC_{50} results for HSV-2 viral antigens, specifically for HSV-2, clinical specimen #4 (TBT-IC50 = 0.25 μg/ml);

FIGURE 9 represents the analytical IC_{50} results for HSV-2 viral antigens, specifically for clinical specimen #5 (TBT-IC_{50} = 0.5 μg/ml); and

FIGURE 10 represents the analytical IC_{50} results for HSV-2 viral antigens, specifically for clinical specimen #6 (TBT-IC50 < 0.10 μg/ml).

DETAILED DESCRIPTION OF THE INVENTION

The invention pertains to methods and pharmaceutical compositions for preventing or treating viral infections in a mammal, and the dermal or mucosal lesions or tumors caused by viral infections in mammals including, but not limited to, human herpesvirus infections (HHV), and more preferably primary or recurrent HSV infections.

In one aspect of the invention, primary and recurrent lesions, sores, or tumors of the skin and mucosa are treated with a topical composition comprising an effective amount of an anti-microtubule agent to a human suffering from herpesvirus infections. The area to be

treated may include, the lips, eyes, mouth, genital and anal area, and other areas accessible to topical administration, which may be the site of a herpes lesion, sore, or tumor.

In a second aspect of the invention relates to a method of treating or preventing herpesvirus infections using pharmaceutical compositions that contain an effective dosage of an anti-microtubule agent which is administered to a patient suffering from viral infections, or at risk for contracting viral infections, in order to treat or prevent the viral infection in the patient.

In a third aspect of the invention, primary and recurrent infections caused by a herpesvirus that may or may not be associated with lesions, are treated with a pharmaceutical composition or a combination of pharmaceutical compositions that is used for regional or systemic administration of an inhibitory effective amount of an anti-microtubule agent contained in the pharmaceutical composition or anti-microtubule agents contained in the combination of pharmaceutical compositions. These types of infections would include viral caused neonatal diseases, encephalitis diseases, and respiratory distress syndrome or acute-onset bronchospasm diseases.

The pharmaceutical compositions of the invention contain an antiviral agent. For the purpose of this invention, the antiviral agent is any molecule from the pharmaceutical composition that interferes with the normal structure and function of microtubule dynamics within a host cell. The antiviral agent interferes with the viability, production, or activity of tubulin proteins within a host cell, including the promotion or inhibition of microtubule polymerization, or the promotion or inhibition of microtubule disassembly. Additionally, the antiviral agent may also exhibit inhibitory effects upon MAPs that mediate the transport of viral particles including a viral genome within the host cell.

The scope of the invention includes any pharmaceutical composition that modifies the activity or effect of microtubule-mediated cytoplasmic transport of viral particles, either directly or indirectly, in decreasing the permissiveness of cells to virus infection. The specific embodiments of this invention as described herein are not intended to limit the applicability of the principles involved. Those skilled in the art are aware that there are, or may be, other means of modifying the activity of microtubules.

The present invention provides pharmaceutical compositions which may contain between 0.005% and 30%, (weight percentage) of the antiviral agent as described above and of one or more of a plurality of pharmaceutically acceptable excipients. Among the plurality of pharmaceutically acceptable excipients include lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, alginates, tragacanth, gelatin, calcium

silicate, mircrocrystalline cellulose, polyvinylpyrrolidone, cellulose, sterile water, syrup and methyl cellulose.

The pharmaceutical compositions can additionally include: lubricating agents such as talc, magnesium stearate and mineral oil; wetting agents; emulsifying and suspending agents; preserving agents such as methyl-benzoates and proplyhydroxy-benzoates; sweetening agents and flavoring agents.

The invention can be formulated so as to provide quick, sustained or delayed release of the antiviral agent after administration to the patient by employing procedures known in the art. Other components may be added to the pharmaceutical composition based upon the related drug delivery system, to improve the pharmacokinetics or pharmacodynamics of the composition.

In making the compositions of the invention, the antiviral agent is usually mixed with and/or diluted by, one or more excipients. Alternatively, the antiviral agent may be enclosed within a carrier, in the form of a capsule or other container. As the excipient may be solid, semi-solid, or liquid, the resultant composition may also be solid, semi-solid, or liquid.

The pharmaceutical compositions of the present invention can include other active ingredients. For instance, drugs that are commonly used to treat herpes, such as nucleoside analogs, or pain relieving drugs such as acetaminophen may be added to the composition. Additionally, agents or chemical additives that enhance the antiviral activity of the agent, or more than one antiviral agent, can be included in the pharmaceutical composition. It may also be desirable to include one or more penetrating agents, such as dimethyl sulfoxide (DMSO), to convey the active ingredients into the epidermal tissues.

Topical pharmaceutical compositions in the form of an ointment, cream, gel, solution, lotion, emulsion, aerosol, powder, or other topical vehicle, including a sponge, suppository or stick, are designed and prepared such that a therapeutically effective amount of the antiviral agent is brought into contact with diseased tissue. An excipient or carrier may take a wide variety of forms depending on the form of preparation desired for topical administration, which includes rectal or vaginal administration. In preparing pharmaceutical compositions in topical dosage form, any of the usual pharmaceutical media may be used.

To create a viscous ointment, for example, de-ionized water, oil and an emulsifier are intermingled to create an emulsion. An oil suitable for such a purpose is petrolatum registered by the United States Pharmacopia/National Formulary (USP/NF). A wax suitable as an emulsifier is wax registered by the USP/NF. A preservative ointment includes USP/NF registered methyl paraben or propyl paraben and humectants, such as propylene glycol.

Solid compositions such as tablets, pills and capsules may be prepared by mixing the antiviral agent with an excipient so that the antiviral agent is evenly distributed throughout the composition, thus making it possible to subdivide the composition into equally effective doses.

The antiviral compositions of the present invention may be incorporated into liquid forms to be administered orally or by injection including aqueous or non-aqueous solutions, syrups, aqueous or oil suspensions and emulsions comprising edible oils, such as corn oil, as well as tinctures and similar pharmaceutical vehicles.

Compositions for inhalation may include solutions and suspensions in pharmaceutically acceptable, aqueous or organic solvents or powders. The liquid or solid compositions may contain pharmaceutically acceptable excipients as described herein. Administration of the compositions through the oral or nasal respiratory tract is preferred for local or systemic effect.

Although certain preferred embodiments of the invention have been described herein, these are not meant to limit the invention, which covers all alternatives, modifications and equivalents as may be included within the scope of this invention. The preferred embodiments presented herein are represent sent the most useful embodiments of the invention, as well as a description of the underlying principles and conceptual aspects of the invention.

Treatment with the pharmaceutical compositions of this invention may begin when a patient has been exposed to a virus, when symptoms of infection, such as epidermal lesions, are apparent in a patient, or when a patient is diagnosed with an active viral infection. Treatment should be continued until risk of infection is over or until the active symptoms of the viral infection have subsided.

As will be apparent to those skilled in the art, various modifications, adaptations and variations of the preceding and foregoing specific disclosure can be made without departing from the scope of the invention claimed herein. The following examples are intended only to illustrate and describe the invention rather than limit the claims which follow.

The herpes simplex viruses, including HSV-1 (oro-facial) and HSV-2 (genital herpes), present a serious problem for millions of people worldwide. To the best of applicant's knowledge, until now, an effective, economical, and readily available topical treatment has not existed.

Herpes simplex virus is a common, recurrent, and chronic infection. It is estimated that at least 75% of the world's population has been infected with HSV-1 and more than 20% with HSV-2. Although the majority of cases are asymptomatic, chronic outbreaks of lesions

are very common, usually occurring in mucous membrane areas and the surrounding skin. The most common of these lesions occur on the lips or face and are commonly referred to as "cold sores" or "fever blisters." Genital herpes lesions occur on the genitals and buttocks and are particularly troubling because of their possible role in contributing to the spread of HIV.

Herpes lesions first appear as an area of irritation (an itching or burning sensation) known as the prodromal stage. Within a few hours, these lesions develop into small vesicles or blisters. Typically, these vesicles soon rupture and form shallow ulcerations which may scab over and heal in about ten to twenty days. The ruptured vesicles may also cause secondary infections and spread the virus to the surrounding tissue.

After initial exposure to the herpes simplex virus, the host develops antibodies that can maintain the virus in a latent state. Despite the presence of antibodies, the latent virus may be reactivated by stress, exposure to sunlight, fever, hormonal changes, menstruation, and trauma. Eruptions can occur randomly and may persist for weeks.

Research supported in part by the National Institute of Allergy and Infectious Diseases (NIAID), and conducted by virologists at the University of Chicago and the University of Alabama, has demonstrated that compounds comprising at least one anti-microtubule agent possess clinically significant anti-viral properties that specifically inhibit replication of HSV-1 and HSV-2 *in vitro*.

There is currently no known cure for herpes simplex virus infections. However, a topical therapy that delivers clinically-demonstrated, anti-viral compositions to the affected area, inhibiting viral replications in the lesions, accelerating healing of the existing lesion, and preventing the spread of secondary infections, would be of enormous benefit to the herpes sufferer.

In one embodiment, the present invention provides compositions useful for treating diseased, biological tissue, such as the epidermis or mucous membranes, in a mammal. The methods and compositions of the present invention are effective in treating viral infections, dermal or mucosal lesions, inflammation, or tumors associated with said viral infections. The compositions of the present invention include at least one anti-microtubule agent. The anti-microtubule agent can be a naturally occurring compound, a semi-synthetic compound, or can be an entirely synthetic compound that is chemically synthesized by any means known to those skilled in the art.

Examples of compounds that have been identified as anti-microtubule agents include, but are not limited to, taxanes and taxoids, discodermolide, epothilones A and B, eleutherobin, taccalonolide, colchicine, colcemid, demecolcine, the vinca alkaloids including vincrisitine, vinepidine, vindesine, vinblastine, vinorelbine, desformyl vincrisitine, desacetyl

desformyl vincristine, and vinflunine, phomopsin A, ustiloxins, cryptophycins, halichondrins, estramustine, rhizoxin, nocodazole, and any analogues or derivatives of any of the above.

A presently preferred composition of the present invention is formed from an extract from the Pacific Yew tree (*Taxus brevifolia*) combined with virgin olive oil and beeswax. The *T. brevifolia* extract is extracted by the method set forth in Example 1 herein and combined with olive oil at a ratio of about 1:1. The ethanol and water (from the extract) are completely evaporated before combining with the beeswax at a ratio of about 6:1. High Performance Liquid Chromatography (H.P.L.C.) analysis of the foregoing *T. brevifolia* extract revealed the presence of 8.1 μg/ml paclitaxel, 77.87 μg/ml cephalomannine, and 623.79 μg/ml 10-deacetyl-7-xylosyltaxol, plus some other taxanes present in minor amounts. Preferably, the compositions of the present invention are topically applied in the form of an ointment, salve or lotion to the site of disease. Compositions of the present invention can be mixed with other physiologically acceptable components, such as carriers, stabilizers or antioxidants, to form an ointment, salve or lotion having desirable physical and chemical properties, and consistency. *See,* Remington's Pharmaceutical Sciences, 16th Edition, Osol, A., Ed (1980).

Preferably, compositions of the present invention include at least one anti-microtubule agent in an amount of from about 0.005% to about 30% of the total weight of the composition. Preferably, the compositions of the present invention also include a natural oil such as, but not limited to, olive oil, mineral oil, corn oil, sunflower oil, peanut oil, and fish oil. Preferably, the compositions of the present invention also contain a wax such as, but not limited to, beeswax, U.S.P. Carbowax 5000®, U.S.P. Carbowax 600® (the foregoing Carbowax® products are manufactured by Union Carbide Corporation, World Headquarters, 39 Old Ridgebury Road, Danbury, and CT. 06817-001) and petrolatum.

In addition to the foregoing components, compositions of the present invention can include additional ingredients including, but not limited to, analgesics and anesthetics.

The compositions of the present invention, when applied topically, soothe the discomfort associated with viral lesions and other epidermal conditions, prevent the lesion from cracking or bleeding, reduce the time to healing, and prevent the spreading of viral infections by effectively inhibiting viral replication.

In another embodiment, the present invention includes a method of treating diseased biological tissue, such as the epidermis or mucous membranes, in a mammal. The method of the present invention includes the step of contacting a diseased biological tissue, such as the epidermis, with a composition of the present invention containing an amount of an anti-microtubule agent, or related compound, that is effective to ameliorate the disease symptoms.

The methods and compositions of the present invention are effective in treating viral infections, dermal or mucosal lesions, inflammation, or tumors associated with said viral infections. Examples of viral infections that can be treated using the compositions and method of the present invention include, but not limited to, HSV-1, HSV-2, HSV-6, HSV-7, HSV-8, VZV, CMV, EBV, and PPV.

The compositions of the present invention should be applied directly to the affected portion of the mammalian body, such as the epidermis. Preferably, in the practice of the method of the present invention, the quantity of a composition of the present invention that is applied to an affected bodily surface is sufficient to cover the affected area. A sufficient quantity of a composition of the present invention should preferably be reapplied as often as is necessary to keep the affected area covered until the condition has completely cleared. In the case of viral infections such HSV-1 or HSV-2, a composition of the present invention should be applied at the very first (prodromal) indication of symptoms (*i.e.*, burning, itching, or tingling sensations). Such early application will, in many cases, prevent lesions from fully developing or spreading, thus significantly limiting the time to healing, discomfort, risk of further infection to self and others, and risk of infection from other opportunistic viruses such as HIV.

The following examples merely illustrate the best mode now contemplated for practicing the invention, but should not be construed to limit the invention. Examples 1 and 2 describe methods of manufacture of various forms of the preferred embodiment. Examples 4-6 describe the clinical effectiveness of preferred embodiments applied to patients infected with HSV-1 and HSV-2. Examples 7 and 8 demonstrate the specific anti-viral activity that *Taxus brevifolia Tinctures* (TBT) exhibits in various viral infected cell culture systems.

EXAMPLE 1

Extraction of Naturally-Occurring Taxanes from Yew

Needles and branches of yew tree species are harvested by pruning the terminal branch tips of the selected species in such a way as to encourage new growth and preserve the tree for future harvesting, thus maintaining the existing biomass as a fully renewable resource. The material is then milled in order to increase the amount of exposed surfaces and render the material more compact. One part (by weight) of the material is placed in a suitable container and saturated with two parts (by weight) of ethanol (or other suitable solvent such as isopropyl alcohol, butanol, or methanol in concentrations ranging from 5% to 100%). The resulting mixture is allowed to macerate in the solvent for a specific time

(typically 7 to 14 days) until the material is exhausted of its constituents, and then is hydraulically pressed and filtered to remove the residue of plant material.

EXAMPLE 2

Second Exemplary Method for Extraction of Naturally-Occurring Taxanes from Yew

The raw materials are harvested as described in Example 1 and placed in a columnar percolator. The material may be pre-moistened for several hours in a fraction of the solvent and then passed through a coarse sieve and lightly packed in the chamber, with a wad of gauze below and filter paper above. The drain is closed and sufficient solvent is added to cover the material. The vessel is then covered and allowed to macerate for approximately 24 hours. The drain is then opened and fluid is allowed through at the rate of 10 to 30 drops per minute, solvent being added to the top as needed until the material is exhausted. The material is then hydraulically pressed to extract any remaining fluid which is then added to the percolate.

EXAMPLE 3

Effectiveness of the Compositions of the Present Invention in Treating Cold Sores

An adult female, suffering from severe, recurrent HSV-1 infections, applied the presently preferred composition of the invention to a labial infection (cold sore) after it had developed into a large blister (the presently preferred composition of the invention is a salve prepared from a *T. brevifolia* extract, prepared by the method set forth in Example 1 herein, which is combined with olive oil at a ratio of about 1:1. The ethanol and water (from the extract) are completely evaporated before combining *T. brevifolia* extract and olive oil with beeswax at a ratio of about 6:1.). The composition was reapplied regularly as needed to keep the blister covered. The blister disappeared within 24 hours and was replaced by healthy tissue. Several months later, the subject experienced prodromal symptoms (*i.e.*, tingling and itching) and applied the compound immediately. Again, the composition was reapplied regularly for 24 hours. The infection did not progress further and produced no lesion or other evidence of infection.

EXAMPLE 4

Effectiveness of the Compositions of the Present Invention in Treating Genital Herpes in a Female Subject

An adult female, suffering from mild, recurrent HSV-2 infections that typically produced lesions on the genitalia lasting approximately 7 days, applied the presently preferred composition of the present invention to the affected parts immediately upon experiencing prodromal symptoms. The infection did not progress further and produced no lesions or other evidence of infection.

EXAMPLE 5

Effectiveness of the Compositions of the Present Invention in Treating Genital Herpes in a Male Subject

An adult male, suffering from moderately severe, recurrent HSV-2 infections that typically produced lesions on the genitalia lasting approximately 7 to 10 days, applied the presently preferred composition of the invention to the affected parts immediately upon experiencing prodromal symptoms. The composition was reapplied regularly as needed to keep the affected area covered. The prodromal symptoms were resolved within 48 hours. The infection did not progress further and produced no lesions or other evidence of infection.

EXAMPLE 6

Effectiveness of the Compositions of the Present Invention in Treating Genital Herpes in a Male Subject

An adult male, suffering from moderately severe, recurrent HSV-2 infections that typically produced lesions on the genitalia lasting approximately 8 days, applied the presently preferred composition of the invention to the affected parts immediately upon experiencing prodromal symptoms. Some lesions did appear and the composition was reapplied regularly as needed to keep the affected area covered. All symptoms were resolved within 4 days.

EXAMPLE 7

Screening Assays for Activity of TBT (ARB ID# 980332) Against HSV-1, HSV-2, CMV, VZV, and EBV

The data below, discloses the results of ELISA assays demonstrating the effectiveness of the *Taxus Brevifolia Tinctures* (TBT) extracts (Lot ARB-ID# 99-332) of Example 1 against Herpes Simplex Virus-1 (HSV-1).

General Approach for Determining Antiviral Activity and Toxicity

A. Screening Assays for Activity Against HSV-1, HSV-2, CMV, and VZV

All the screening assay systems utilized have been selected to show specific inhibition of a biologic function, i.e., cytopathic effect (CPE) in susceptible human cells. In the CPE, inhibition assay, drug is added 1 hr prior to infection so the assay system will have maximum sensitivity and detect inhibitors of early replicative steps such as absorption or penetration as well as later events. To rule out non-specific inhibition of virus binding to cells all compounds that show reasonable activity in the CPE assay are conformed using a classical plaque reduction assay in which the drug is added 1 hr after infection. In the case where a compound blocks attachment, it will show up positive in the CPE assay, but may be negative by plaque assay. In this case, the plaque assay is repeated with drug being added

prior to viral infection. Using this approach, we have been able to identify compounds that inhibit virus absorption. These assay systems also can be manipulated by increasing the pretreatment time in order to demonstrate antiviral activity with oligodeoxynucleotides and/or peptides and by delaying addition of drug after infection, information regarding which step in the virus life cycle is inhibited (i.e., early vs. late functions) can be gained.

1. Efficacy. In all the assays used for primary screening, a minimum of six drug concentrations were used covering a range of 100 μg/ml to 0.03 μg/ml, in 5-fold increments. From these data, we calculate the dose that inhibits viral replication by 50% (effective concentration 50; EC_{50}) using the computer software program MacSynergy II by M.N. Prichard, K.R. Asaltine, and C. Shipman, Jr., University of Michigan, Ann Arbor, Michigan.

2. Toxicity. The same drug concentrations used to determine efficacy are also used on uninfected cells in each assay to determine toxicity of each experimental compound. The drug concentration that is cytotoxic to cells as determined by their failure to take up a vital strain, neutral red, (cytotoxic concentration 50; CC_{50}) was determined as described above. It is very important to determine the toxicity of new compounds on dividing cells at a very early stage of testing. We have found that a cell proliferation assay using human foreskin fibroblasts (HFF) cells is a very sensitive assay for detecting drug toxicity to dividing cells and the drug concentration that inhibits cell growth by 50% (IC_{50}) is calculated as described above. In comparison with four human diploid cell lines and vero cells, HFF cells are the most sensitive and predictive of toxicity for bone marrow cells.

3. Assessment of Drug Activity. To determine if each compound has sufficient antiviral activity that exceeds its level of toxicity, a selectively index (SI) is calculated according to CC_{50}/EC_{50}. This index, also referred to as a therapeutic index, was used to determine if a compound warrants further study. For these studies, a compound that had an SI of 10 or greater was evaluated in additional assay systems.

B. Confirmation of Antiviral Activity and Toxicity for HSV, CMV, and VZV

1. HSV-1 and HSV-2. Compounds that showed activity in the CPE-inhibition assay was confirmed using the plaque reduction assay as described in an earlier section. Susceptibility of additional virus strains including both lab passaged and clinical isolates was determined for selected compounds. A battery of ACV resistant HSV strains were also utilized.

2. CMV. Compounds that have activity in the CPE-inhibition assay were confirmed using the plaque reduction assay in HFF cells. A variety of laboratory, clinical, and GCV resistant isolates are also available for testing.

3. VZV. Compounds were tested for activity in a plaque reduction assay. A battery of laboratory, clinical, and ACV-resistant isolates are available.

4. Toxicity. In addition to the toxicity component incorporated into each assay system, a standardized cell cytotoxicity assay using a vital strain uptake (Neutral Red) was performed using 7 days of drug exposure to confluent non-dividing cells. This assay measures direct cell killing (CC_{50}). Inhibition of cell growth (IC_{50}) can also be determined by treatment of proliferating cells and then assessing the amount of dye uptake.

C. Assay Systems for Determining Antiviral Activity Against EBV and Toxicity to Lymphoblastic Cells

1. Superinfection of susceptible Burkitt's Lymphoma (BL) cells with P3HR-1 virus followed by analysis of specific EBV gene product expression using monoclonal antibodies provides a convenient and repeatable system of evaluate inhibition of EBV gene expression during early and late stages of the virus replication cycle. We can evaluate diffuse (D) and restricted (R) early antigens (EA) as well as viral capsid antigen (VCA) by fluorescence microscopy and by fluorescence flow cytometry.

2. Screening Assay for EBV Activity. The initial system to be used to determine antiviral activity against EBV will be VCA production Daudie cells using an immunofluorescence assay (IFA). As in all the other assays, six concentrations of drug covering a range of 100 μg/ml to 0.03 μg/ml will be utilized. Using the results obtained from untreated and drug treated cells an EC_{50} can be calculated. Selected compounds that have good activity against EBV VCA production without toxicity will be tested for their ability to inhibit EBV DNA synthesis.

3. Toxicity. In each assay system utilized, drug treatment of uninfected cells is incorporated to obtain as much toxicity data as possible.

4. Confirmation of drug activity against EBV DNA production using in situ DNA hybridization assay. All compounds that have an SI > 10 in the screening assay or ones selected by the project offer will be confirmed in a hybridization assay that measures the amount of EBV DNA produced by P3HR –1 infected cells. As in all other assay systems utilized, a wide range of drug concentrations will be utilized so an accurate EC_{50} can be calculated. Uninfected control cells treated with drug will also be utilized as another measure of drug toxicity.

a. Infection and drug treatment: 10^6 cells/tube are infected with EBV at a dilution of 1:40. After incubation for 45 minutes at 37° C, 3 ml of RPMI, a cell culture media, is added and the cells pelleted by centrifugation. The supernatant was then discarded and the cells resuspended in 4ml of RPMI needing containing various concentrations of drug. After incubation for 48 hours, the cells are counted in each tube, washed with PBS and spotted on slides. The slides are left to air-dry overnight and then fixed in acetone for 10 minutes at room temperature.

b. DNA hybridization: The biotin labeled EBV probe is added to each spot and the slide is covered with a glass overslip. The slide is then heated on a hot plate at 95° C for three minutes. After heating, the slide is left to sit at room temperature for 20 minutes, for the DNA to anneal. The overslips are then removed and the Post Hybridization Reagent is added to each spot. After incubation for 10 minutes and rinsing with washing buffer, Detection Reagent is applied. This is left on for 20 minutes at room temperature and then washed off with washing buffer. Chromagen Substrate Solution is added and incubated for 10 minutes at room temperature. Washing buffer is used to rinse it off, and the slides are counter stained for 30-60 seconds with fast Green stain. The slides are then rinsed with deionized water and mounted with water.

c. Reading and calculation of results: The slides are viewed in a light microscope under a magnification of 100-400. Positive cells appear as pink or red spots. All the cells are counted in several fields. The fraction of red spots in the total number of cells counted multiplied by 100 reflects the percent of hybridization.

EXAMPLE 8

ELISA Testing Of HSV Susceptibility To TBT

The data below discloses the results of ELISA assays demonstrating the effectiveness of the *T. brevifolia* tincture extracts of Example 1 against Herpes Simplex Virus (HSV).

Susceptibility Testing by ELISA (Enzyme Linked Immunosorbent Assay)

HFF cells were inoculated into 96-well microtiter trays at a density of 7 X 10^3 cells per well. The plates were incubated at 37° C in 5% CO_2 until the cells were confluent, usually three days. Sixty wells of each plate were used: six uninfected cell control wells, six virus-infected control wells without drug, and six replicates of eight dilutions of the drug. Dilutions of each virus were prepared in minimal essential media (MEM). The growth medium was removed from all wells and 50 µl of MEM was added to the cell control wells and 50 µl of virus inoculum with a multiplicity-of-infection (MOI) ~ 0.05, was added to the

remaining wells. The virus was allowed to absorb for one hour at 37° C. The inoculum was removed and 100 µl of MEM was added to the cell control wells and the virus control wells. Eight dilutions of *Taxus brevifolia Tincture* (TBT) were prepared in MEM and 100 µl of the diluted drug was added to the remaining wells beginning at a 4.0 µg/ml through a 0.1 µg/ml of the drug. All plates were incubated at 37° C in 5% CO_2.

After incubating for 48 hours, the plates were examined using an inverted phase contrast microscope to insure that viral CPE was present in the virus control wells and to score the CPE in all wells of the plate including the virus control wells and all drug dilution wells. Each row of wells was scored from 0 to 4+ and 4+ indicated that all cells showed CPE. This was done to insure that the inhibition of CPE correlated with the quantitative ELISA results. The medium was then removed from all microtiter wells and 100 µl of a blocking solution consisting of 0.5% bovine serum albumin (BSA) in phosphate buffered saline (PBS), pH 7.2, was added to each well for 30 min. at room temperature. The blocking solution was removed, the cells were fixed by adding 100 µl of ethanol/acetone (95:5, v/v) to each well and the plates were placed at –20° C for 30 min. Each well was washed four times with 200 µl of wash solution (PBS containing 0.5% BSA and 0.05% Tween 20).

The antibodies used in ELISA were obtained from Dako Corporation, Carpinteria, California, and were prepared by immunizing rabbits with an antigen prepared by sonication and extraction of HSV-1 or HSV-2 infected rabbit cornea cells. All the virion proteins were present in the antigen preparation used to produce the antibody. To determine the inhibitory concentration (IC_{50}), the rabbit polyclonal antibody to HSV-1 or HSV-2 conjugated to horseradish peroxidase was diluted in PBS containing 10% normal rabbit serum. A volume of 100 µl of the antibody was added to each well and the plates were incubated at 37° C for two hours. The antibody was removed and the wells were washed four times as before. The enzyme substrate, 3, 3', 5, 5' – tetramethylbenzidine (TMB, Sigma, ST. Louis, MO) was added to each well and the plates were incubated at room temperature for 3-4 minutes. The O.D. was determined for the uninfected cell control wells, the virus control wells, and each drug dilution. The percent change in O.D. was calculated as follows: (average drug sample O.D. – average cell control O.D.)/(average virus control O.D. – average cell control O.D.) X 100. The IC_{50} is defined as the dilution of antiviral compound that produces a 50% or greater reduction in the O.D. of the colored substrate product.

While the preferred embodiment of the invention has been illustrated and described, as noted above, many changes can be made without departing from the spirit and scope of the invention. Accordingly, the scope of the invention is not limited by the disclosure of the preferred embodiment.